APPENDIX A

age. When the effect of these reductions is correlated with outcome, there is a strong suggestion of a negative impact.[120,121] In premenopausal women, the differences in relapse-free survival at both low and high doses of CMF are statistically significant. The importance of dose effect was further confirmed by a large study in which a survival benefit was observed as a result of increasing dose intensity in the adjuvant chemotherapy for women with stage II, node-positive breast cancer.[122]

An increase in the dose intensity represents one approach to improve on the effect of specific drugs or drug combination, but it may not be useful in all clinical circumstances. In the setting of large tumor burdens, the dose-response curve tends to shift to the right. At the low end of the curability curve (i.e., in the presence of the highest tumor burdens), an increase in dose intensity may not improve treatment outcome, as the dose-response curve is flat, but most often leads to unacceptable host toxicity. In addition, increasing the dose intensity of drug regimens that are already associated with curing nearly 100% of a subset of patients would not be expected to be of clinical benefit. Such a scenario would hold for the treatment of germ cell cancer using the cisplatin, etoposide, bleomycin combination and for Hodgkin's disease, using either the mechlorethamine, vincristine (Oncovin), procarbazine, and prednisone regimen; the doxorubicin, bleomycin, vinblastine, and dacarbazine regimen; or regimens derived from them, such as BEACOPP (see Chapter 45.3). However, for most drugs, there appears to be a threshold dose that produces clinical response. The success of high-dose chemotherapy programs with stem cell support in refractory lymphomas, breast cancer, childhood sarcomas, and neuroblastomas suggests that maximizing dose intensity can improve response rates or cure in drug-responsive tumors.

Frei et al.[123] and Hryniuk et al.[124] have proposed the term *summation dose intensity* to reflect the relationship between dose and combination chemotherapy. As part of this concept, they suggested that the final outcome of either a high-dose or combination treatment must be related in some manner to the sum of the dose intensities of all the agents used in that treatment. The intrinsic chemosensitivity of a given tumor is critical for treatment success. An active agent is defined as one that, when used alone, is associated with at least a 30% response rate for a given tumor. It is now well appreciated that for almost all malignancies, a combination regimen incorporating at least three active drugs is necessary for cure. In the case of childhood leukemia, the cure rate increases linearly when the number of active drugs increases from three to seven. The critical issue for this concept is that all active agents must be used at their full therapeutic doses. However, until the advent of the various cytokine growth factors and autologous or peripheral stem cell transplantation (or both), the effective administration of maximal doses of chemotherapy has not been possible. Although the concept of summation dose intensity is not new, it does offer a unified approach for the careful design and interpretation of clinical trials.

IN VITRO DRUG-RESPONSE ASSAYS

Several methods have been developed since the 1950s to determine the *in vitro* drug sensitivities of human tumor cells to various anticancer agents.[125–139] The advent of reliable *in vitro* drug-response assays has raised the possibility of selecting effective anticancer agents to be used either alone or in combination to treat a patient's individual tumor. In this setting, identification of agents with an extremely low probability of response makes it possible to eliminate the use of such agents and thus their potential for adverse events. A number of methods have been used to investigate the sensitivity of tumors and tumor cell lines, including clonogenic, differential staining cytotoxicity assay; colorimetric, rapid ^{3}H-thymidine incorporation assay; and chemotherapeutic treatment of athymic nude mice with human tumor xenografts. Fruehauf and Bosanquet[140] reviewed the correlation between the results of *in vitro* sensitivity testing and a patient's tumor response to chemotherapy and, in general, they found an overall sensitivity of 85% and an overall specificity of 80%.

In the mid-1950s, Black and Spear[134,135] were the first to report the use of an *in vitro* assay to predict patient response. Their studies compared the *in vitro* activity of aminopterin with its clinical response. Their assay technology was based on the colorimetric detection of viable cells using a substrate for mitochondrial succinate dehydrogenase. Although the predictive accuracy of their results was not particularly strong, the development of the clonogenic stem cell assay in the 1970s brought *in vitro* testing of solid tumors into the mainstream.[137] However, the results of these studies indicated that there were significant technical issues to overcome.[138,139] As illustrated in Table 17-4, further work to improve on the technology led to a variety of techniques and approaches with a pronounced ability to identify drug resistance accurately. The major distinction among the differing assay methods is the end point used to measure cell viability. Assay end points include colony growth from single stem cells, incorporation of tritiated thymidine, microscopic examination of cells with vital dyes, mitochondrial enzyme activity, cytosolic esterase activity, and adenosine triphosphate content. Given the variety of assay types, it is remarkable that the predictive accuracy for the identification of chemosensitivity for most of these approaches appears to be at least 90%. Several issues should be considered when evaluating an assay technology (Table 17-5).[125,127,140]

The clonogenic assay evaluates the ability of chemotherapeutic agents to inhibit tumor stem cell proliferation in agarose, a medium that precludes proliferation of nontransformed cells.[133,134] Most of the *in vitro* drug-response techniques use similar methods for tumor preparation. Solid tumors are disaggregated into suspensions of multicellular clumps with scissors and by passing the fragments through mesh or by stirring tissue fragments with collagenase. Single-cell suspensions then are generated by passing the cellular aggregates through high-gauge needles.[138,138] Cell suspensions are incubated with drug for 1 hour, rinsed, and plated on an agar base with growth media. After a period of 14 to 28 days, the number of colonies that have grown from the treated cells is compared with the number of colonies from untreated control cells. The fraction of control growth provides an index of drug activity. Studies by the National Cancer Institute and the Southwest Oncology Group indicate that the assay is reproducible among multiple laboratories.[138] Problems with assay interpretation arising from the initial plating of small cell clumps were overcome with the use of chromomycin A3.[138,139]

The conventional clonogenic stem cell assay has suffered from a relatively low success rate (<50%) of specimens yielding results, rendering it difficult to accrue adequate numbers of

TABLE 17-4. Correlations of *In Vitro* Test Results with Patient Response

Assay Type	*Patients*	*TP*	*TN*	*FP*	*FN*	*Predictive Accuracy*[a] +	*Predictive Accuracy*[a] −	*Sensitivity (%)*[b]	*Specificity (%)*[c]
Clonogenic	2300	512	1427	226	135	69	91	79	86
5 d thymidine	494	123	432	119	20	51	92	86	66
3 h thymidine	171	90	40	21	20	81	67	82	66
DiSC	510	247	175	72	16	77	92	94	71
MTT	326	187	74	37	28	83	73	87	67
ATP	129	74	37	6	12	93	76	86	86
FCA	333	154	116	52	11	75	91	93	69
Total	4263	1387	2101	533	242	72	90	85	80

ATP, adenosine triphosphate; DiSC, differential staining cytotoxicity; FCA, fluorescent cytoprint assay; FN, patients who are resistant *in vitro* but respond clinically; FP, patients who are sensitive *in vitro* but resistant clinically; MTT, tetrazolium dye; TN, patients who are resistant *in vitro* and do not respond to chemotherapy; TP, patients who are sensitive *in vitro* and respond to therapy.

[a]Predictive accuracy: + indicates TP/(TP + FP), percentage of patients with sensitivity in the test who respond; − indicates TN/(TN + FN), percentage of patients with resistance in the test who do not respond to therapy.

[b]Test's ability to detect clinically responsive patients.

[c]Test's ability to detect clinically unresponsive patients; clinical response is greater than or equal to a 50% reduction in assessable disease.[85]

Note: Summary of clinical correlations is pooled from individual studies referenced in text.

patients into clinical trials.[138] Although this factor initially dampened enthusiasm for this approach, a significant number of clonogenic assays (>2500 cases) have now been performed by various groups, with an overall positive predictive value of 69% and a negative predictive value of 91% (see Table 17-4).[120]

Tritiated thymidine incorporation, as an assay end point, was introduced in part to eliminate the problem of discriminating between true colony growth from a single cell and from a clump of cells plated at the outset. This technique also decreased the assay time from more than 14 days to less than 1 week and was associated with an improved success rate of diagnostic yield to 85%.[125–127,141,142]

Processing and plating of the tumor for the thymidine assay is similar to that for the clonogenic assay. However, in the thymidine assay, small clumps rather than single cells are preferred to maintain cell-cell interactions. In addition, cells are grown in an agar suspension, which allows tumor growth *in vitro* to recapitulate the three-dimensional *in vivo* morphology. Cell-cell interactions resulting from three-dimensional growth may be critical for the detection of acquired drug resistance, which can be missed in monolayer cultures.[143]

In contrast to the clonogenic assay, prolonged drug exposures are utilized in the thymidine-based system. Tumor suspensions are continuously exposed to drug for 5 days, and tritiated thymidine is added during the final 48 hours of the assay to label proliferating cells. Determination of drug action is based on a comparison of the incorporation of labeled thymidine by untreated controls with incorporation by the groups treated with different drugs. Clinical correlations obtained using this assay technique demonstrate a reasonable overall predictive accuracy (72%) and indicate that it is an accurate predictor of drug resistance (99%).[126] The prolonged drug exposure in the thymidine assay results in a five- to 20-fold higher concentration × time factor than that used in the clonogenic assay, biasing assay accuracy toward detection of drug resistance. Tumor growth after drug exposure in the thymidine assay is associated with multifold drug resistance, which led the authors of one article to describe it as the "extreme drug resistance assay."[126] Some paclitaxel-resistant tumors identified with this technique have been found to overexpress P170 glycoprotein, suggesting that this assay can be used to identify the activity of specific mechanisms of drug resistance in different tumor histologies.[144]

Another promising assay is the differential staining cytotoxicity (DiSC) assay.[140,145] The DiSC assay relies on the structural integrity of cells. In the DiSC assay, cells are incubated with drugs for 4 days. Dead cells are stained in suspension with fast green dye in the absence or presence of nigrosin, and duck red blood cells are added as an internal standard for counting. The specimen is cytocentrifuged to deliver discs of cells onto microscope slides. Live cells then are stained with either hematoxylin-eosin or Romanowsky stain. The end point of this test is the morphologic identification of tumor cell cytotoxicity as compared with the internal control of fixed duck erythrocytes. The DiSC assay requires more than 10% tumor cells and measures cell kill in both dividing and nondividing tumor cell populations. Microscopic identification of the cell population renders it possible to determine the differential kill of normal and tumor cells,

TABLE 17-5. Factors Influencing the Utility of the *In Vitro* Assay

Tumor heterogeneity: Is the assay end point selective for malignant cells versus stromal cells?
Is the assessability rate greater than 80%?
Have the assays been correlated with clinical response and survival?
Can the tests evaluate all histologic types, or are they restricted to only certain types of tumors?
Are clinically appropriate drugs evaluated in the test?
Does the turnaround time meet clinical requirements?
Is the test information easily interpreted and applied?
Is the test cost-effective?

and this is the therapeutic index for new agents undergoing *in vitro* screening for activity. The DiSC assay (see Table 17-4) offers an overall predictive accuracy of 83%, with a sensitivity of 94% and a specificity of 71%.[125,132,145]

The potential efficacy of individualized chemotherapy selected by *in vitro* drug sensitivity testing for patients with cancer has been reviewed.[146,147] A number of issues seriously limit the widespread use of this approach in the clinic. First, *in vitro* drug sensitivity testing is relatively expensive and time-consuming. Second, the efficient procurement of tumor tissue remains a serious problem. In fact, only two studies, both from the National Cancer Institute, have evaluated the ability to obtain tumor tissue from patients with limited- and extensive-stage small cell lung cancer.[147,148] Tumor tissue was obtained from 80% of patients with limited-stage disease, in contrast to nearly 70% of patients with extensive-stage disease. Third, even with successful procurement of tumor tissue, a host of technical issues limits the ability for efficient and successful drug testing. In fact, of 12 different trials reviewed, only slightly more than one-half of all tumor samples had sufficient cell numbers for drug testing. Finally, only one-third of all patients entered in prospective trials of *in vitro* drug testing were actually treated with an *in vitro* best regimen. In those patients, the response rates appear to be as good as, and perhaps even slightly better than, those achieved with empiric therapy. It is not surprising, then, that when all the clinical studies are taken together, no potential benefit in survival is observed for this approach. Of note, however, is a survival advantage that has been reported, in a small select series of studies, in patients treated with an individualized *in vitro* best regimen.[148]

The reliability of newer *in vitro* assay technologies to identify drug sensitivity suggests that such assays can help the clinician to avoid exposure of patients to the toxicity of drugs with little clinical benefit. Although the promise of an *in vitro* sensitivity assay has not yet been met, there remains value in identifying inactive agents before their administration and eliminating them from drug combinations. These tests render it possible to tailor drug combinations for the individual cancer patient. They also offer a rational stopping point for both the patient and clinician in situations in which the patient's tumor demonstrates extreme resistance to all conventional anticancer agents. An understanding of when to terminate therapy in hopeless situations is as important as any management issue facing the clinician.[149] Although only a few hundred patients have been enrolled to date to evaluate the impact of *in vitro* assay-directed therapy on survival, it is intuitively obvious that there should be a therapeutic advantage in the activity of agents to which a tumor is highly responsive *in vitro* as compared with agents that demonstrate significant *in vitro* drug resistance.[125] Further prospective, randomized studies are needed to define more properly the true role of *in vitro* drug testing in the selection of chemotherapy for cancer patients in the adjuvant, induction, or salvage setting.

Although *in vitro* tissue culture studies serve as an important guide for selecting chemotherapy, they are inadequate at addressing the issues of tumor cell heterogeneity, drug distribution, drug bioactivation, and host toxicity. *In vivo* model systems overcome some of these obstacles, and several have been developed, including the subrenal capsule assay, a semipermeable membrane in the Millipore diffusion chamber as vessels for tumor implantation into mice, and the tumor xenograft model, which is perhaps the most widely used method for drug testing.[140,150–152] However, each of these experimental systems has its unique drawbacks. Recently, a novel system was developed using a semipermeable polysulfone fiber with a molecular weight cutoff of 30 kD. Human cancer cells derived from tissue culture or from patient tumor specimens are injected directly into semipermeable fibers that are then implanted into immunocompetent rats.[153,154] Animals are treated with the given drug and, after a defined period, they are sacrificed, the fibers are recovered, and the remaining viable cells are counted using the trypan dye exclusion method. There are several advantages to this polysulfone fiber model. First, the entire process of tumor recovery, injection, and implantation of fibers, drug treatment, fiber recovery, and cell analysis can be completed in less than 1 week. This short period minimizes the potential waiting time for selection of the optimal drug, thereby rendering this model feasible for application in the clinical setting in treating a patient. Second, the results from this model system are consistent, reliable, and highly reproducible. As many as six to seven fibers can be implanted into an individual rat; thus, each fiber can be injected with the same cell type and the individual rat treated with the same drug. In addition, this reduces the unnecessary expense of using multiple animals for drug *in vivo* testing. Third, because up to six to seven fibers can be implanted into an individual rat, cancer cells derived from different primary tumors can be tested simultaneously for drug sensitivity, a process that can result in greater cost and time efficiency. Further testing and validation are required to determine whether such a novel *in vivo* system can help to individualize and optimize the clinical therapy of cancer patients.

Finally, studies by Waldman et al.[155] have raised concerns regarding the validity of the *in vitro* colony formation assay as a measure of the cytotoxicity of DNA-damaging agents in tumor cells with altered checkpoint response. Using the human colon cancer HCT116 cell line that expresses wild-type p53 and p21 (p21+/+) and a subline that was rendered p21-deficient (p21−/−) by homologous recombination, these researchers tested the effects of γ-irradiation using the *in vitro* colony formation assay and an *in vivo* xenograft model. Of note, they observed no differences in sensitivity to ionizing radiation, as determined by the *in vitro* colony formation assay. However, using the nude mouse xenograft model, they found that tumors derived from the parent p21+/+ cell line were able to survive exposure to ionizing radiation. In contrast, a significant fraction of the tumors deficient in p21 underwent apoptosis and were thus completely cured. Clearly, the *in vitro* assay was unable to detect this significant difference in sensitivity, as both the processes of cell arrest and apoptosis preclude the outgrowth of colonies. Given the critical role of checkpoint status as a determinant of chemosensitivity, these findings are important as they suggest that *in vivo* assays may represent a more relevant model system to compare the effects of anticancer agents.[156] Moreover, such an *in vivo* model may be ideal for testing novel agents that specifically target cell-cycle control and the pathways associated with the process of apoptosis.

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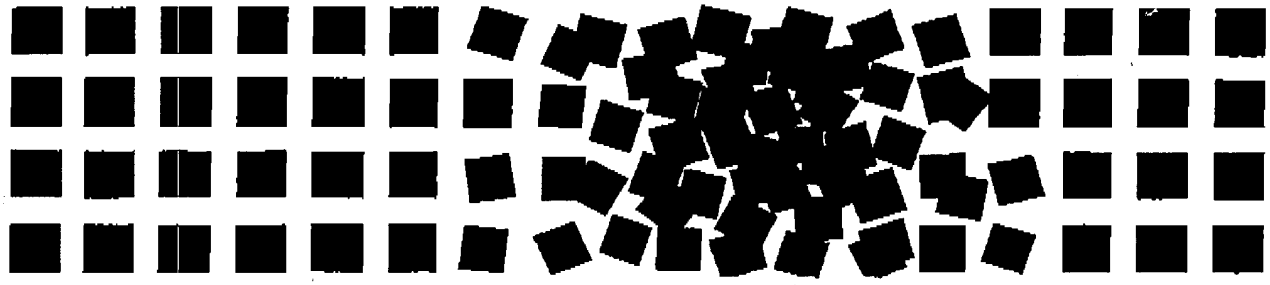

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